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Addition of *N*-Acetylcysteine to Linoleic Acid Hydroperoxide[1]

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ABSTRACT

Catalyzed by 10^{-5}M ionic iron in 80% ethanol, *N*-acetylcysteine added to linoleic acid hydroperoxide, forming a thiobond. Reaction of a specific isomer of the hydroperoxide, 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic acid, and *N*-acetylcysteine, forms a number of products, of which two were identified as addition compounds. One addition product was 9-*S*-(*N*-acetylcysteine)-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid, and the other was 9-*S*-(*N*-acetylcysteine)-10,13-dihydroxy-*trans*-11-octadecenoic acid.

INTRODUCTION

Lipid peroxides degrade proteins by reacting with them to form products by scission (1), protein-protein crosslinking, and covalent bonding with the lipid peroxide (2). Although a secondary product of lipid peroxidation, malonaldehyde, has been implicated as one of the agents responsible for protein crosslinking through Schiff base formation, Roubal (3) showed that radical production is probably the factor most responsible for destruction of amino acid residues. In proteins, among the number of amino acid residues particularly labile are the sulfur amino acids (4,5). Sulfhydryl enzymes are inactivated rapidly by lipid peroxides (6,7) and demonstrate the oxidative sensitivity of the sulfhydryl group. Thiyl radicals are observed in proteins with free sulfhydryl groups after exposure to peroxidized lipid; disulfide bonds do not generate radicals (8). Studies with small thiols, including cysteine, show that thiols are oxidized to disulfide and other products by lipid peroxide (5,9), and the presence of transition metal ions or metalloprotein accelerates oxidation (10,11). Formation of disulfides implies that thiyl radicals are present. Even though thiyl radicals are known to add to olefins (12), no addition products of thiol amino acids and unsaturated lipid peroxide have been characterized.

A number of products result from reaction of linoleic acid hydroperoxide (LOOH) with cysteine as catalyzed by ionic iron (11). These products were characterized as nine oxygenated fatty acids, as well as about an equal number of ninhydrin-reactive lipids (13). To explain the formation of the products, a free radical Fe(II)-Fe(III) redox cycle was proposed by Gardner (14) in which thiyl radicals were generated from cysteine and alkoxy radicals from LOOH. We extended these observations and now report structures of products from reaction of *N*-acetylcysteine and a specific isomer of LOOH, 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic acid (13-LOOH).

METHODS

Reaction Conditions

The reaction solution was 3.2 mM 13-LOOH, 13.0 mM *N*-acetylcysteine (Nutritional Biochemicals Corp., Cleveland, OH), and 10^{-5}M $FeCl_3$ in 80% ethanol. The 13-LOOH was prepared 99+% pure by column chromatography (15). The reaction proceeded for 1 hr at room temperature under a nitrogen atmosphere. Products were extracted with $CHCl_3$ as before (13).

In one experiment a mixture of 79% 13-LOOH and 21% 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (9-LOOH) was synthesized by the method of Gardner et al. (13), and subsequently used in a reaction with *N*-acetylcysteine.

Chromatography

Column chromatography: The product mixture, containing 9-*S*-(*N*-acetylcysteine)-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid (I) and 9-*S*-(*N*-acetylcysteine)-10,13-dihydroxy-*trans*-11-octadecenoic acid (II) (Fig. 1), was methylated with diazomethane, subsequently slurried with 2 g Mallinckrodt SiliCAR CC7 in $CHCl_3$ and applied to a column (inside diameter 2.5 cm) packed with 50 g SiliCAR CC7 in $CHCl_3$. The column was eluted stepwise with 200 ml $CHCl_3$ and 300 ml each of $CHCl_3$ solutions containing 0.5% (v/v), 0.75%, 1.5%, 2%, 3%, and 4% CH_3OH. The 10-ml fractions collected were assayed by thin layer chromatography (TLC). If more separations were required, fractions from the column were purified further by TLC.

TLC: Silica Gel G plates 20 x 20 cm and 250 μ thick were used for analytical and preparative TLC. Quantities in excess of ca. 25 mg

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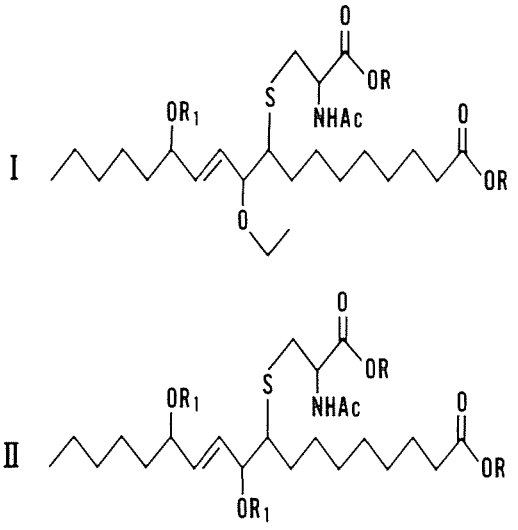


I or II: R and R_1 = H

Ia or IIa: R = CH_3, R_1 = H

Ib or IIb: R = CH_3, R_1 = $Si(CH_3)_3$

FIG. 1. Numerical key and structures of addition products and derivatives from the reaction of *N*-acetylcysteine and 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic acid.

were separated on preparative plates 0.5 mm thick. Triple development (air dried between development) with $CHCl_3$:CH_3OH 98:2 in a tank lined with Whatman 3 MM filter paper served for all preparative separations. Products Ia and IIa were located by the presence of a long wave ultraviolet fluorescence which migrated in a band just in front of them.

Fractions from column chromatography were assayed by TLC. From each fraction a 40 μl sample was spotted, and then the plate was developed with $CHCl_3$:CH_3OH 95:5 in a filter paper lined tank. The spots were charred by heat after spraying the plates with 50% H_2SO_4.

Trimethylsilyloxy (TMS) derivatives, Ib and IIb, were separated from the reagents and other impurities by TLC with hexane:anhydrous ether 1:1 solvent (R_f = 0.08 and 0.13 for Ib and IIb, respectively). Long wave fluorescence indicated the front of the compound. TLC scrapings were eluted with anhydrous ether, care being taken to maintain dryness.

Spectroscopy

Mass spectroscopy (MS) was done on a Dupont 21-492-1 mass spectrometer by probe insertion. Ionization was completed with 70 electron volts.

Infrared (IR) spectra were determined as described previously (13). Nuclear magnetic resonance (NMR) spectra were recorded with a Varian Model HA-100 at 100 MHz or with a Bruker WH-90 operating at 90 MHz. The samples were dissolved in $CDCl_3$ with 1% tetramethylsilane as the internal reference. Sample temperature was 29 C, and absorptions were measured by first order analysis. When compounds containing TMS group(s) were analyzed by 100 MHz NMR, the internal lock was provided by 1% $CHCl_3$. A trace of tetramethylsilane served to measure δ 0 accurately.

Derivatives

Diazomethane was used to esterify (16). Hexamethyldisilazane:trimethylchlorosilane:pyridine 2:1:1 (v/v/v) formed TMS ethers from hydroxyl groups. Standing at room temperature was sufficient to form TMS ethers when the compound was ca. a 10% solution in the reagent.

RESULTS

The Reaction

N-Acetylcysteine was allowed to react with 13-LOOH by introducing 10^{-5} M $FeCl_3$ into the reaction mixture. The reaction was essentially complete within 10-20 min. The products were treated with diazomethane and then separated by column chromatography (Table I). Ia and IIa (Fig. 1) were products in which *N*-acetylcysteine had added to the *cis* double bond of 13-LOOH. Besides Ia and IIa, two other compounds, Ia′ and IIa′, were isolated. By all criteria, except minor differences in NMR spectra, Ia′ and IIa′ were indistinguishable from Ia and IIa, respectively. Because two functional groups had added to vicinal carbons, *erythro-threo* isomerism is the most probable reason there are two isomeric forms for each addition product. Other compounds had spectral characteristics typical of both a fatty ester and an *N*-acetylcysteine methyl ester, indicating that more addition products were present; since these components were minor, they were studied no further. As can be seen from Table I, *N*,*N*′-diacetylcystine dimethyl ester was also observed. On the basis of replicate experiments, product yields vary somewhat, but always I (and I′), II (and II′), and *N*,*N*′-diacetylcystine are present as major products.

Structure Determination

Products I and I′: Products I and I′ were isolated as their methyl esters, Ia and Ia′. Product Ia was isolated by column chromatography (Table I), but Ia′ had to be purified

TABLE I

Column Chromatography[a] of Linoleic Acid Hydroperoxide:*N*-Acetylcysteine Reaction Products after Methyl Esterification

Eluant volume (ml)	Wt (mg)	Identity of component[b]
0-320	83	Mostly fatty esters
320-430	132	Mostly *S*-methyl-*N*-acetylcysteine methyl ester[c]
430-540	20	Unidentified addition products
540-640	32	Ia
640-720	75	Ia, Ia′, and a minor amount of unidentified material
720-900	15	Unidentified addition products
900-1070	33	*N,N′*-Diacetylcystine dimethyl ester and unidentified products
1070-1190	15	IIa
1190-1320	12	IIa′
1320-1790	4	Unidentified

[a]Crude product (550 mg) was applied to a silica column and eluted with $CHCl_3$-CH_3OH.

[b]Ia and IIa are identified in Figure 1. Ia′ and IIa′ were isomers of Ia and IIa that were identical in properties except for minor differences in NMR spectra.

[c]Identified by comparison to a standard synthesized by treatment of *N*-acetylcysteine with diazomethane.

TABLE II

Infrared Spectral Assignments[a] of Addition Products and Their Respective Isomers[b]

Absorption (cm^{-1})		
Ia, Ia′	IIa, IIa′	Assignment
3430(sharp), 1675	3430(sharp), 1665	Amide
3360(broad)	3330(broad)	Amide and hydroxyl
1500	1520	Monosubstituted amide
1085	---	Ether C-O stretch
1020	1020	Secondary alcohol
975	975	Isolated *trans* olefin

[a]Not tabulated are absorption characteristics of fatty esters (e.g., methyl stearate).

[b]Ia′ and IIa′ are isomeric forms of Ia and IIa postulated to be *erythro* and *threo* about carbons 9 and 10 of the fatty ester chain. No difference could be observed in IR spectra between the isomeric forms.

further by TLC after column chromatography.

Spectral data of Ia and Ia′ appear in Table II for IR and in Table III for NMR. The MS of Ib (Fig. 2) is identical to the MS of Ib′ within operating parameters. The MS subtraction ions from Figure 2 are identified in Table IV. Structural assignments are based on these spectral data and on additional experiments outlined below.

The presence of one hydroxyl group was demonstrated by analyzing derivative Ib and Ib′ by NMR. An absorption at δ 0.11 (s, 9 H) indicated the presence of one TMS group. The IR spectra of Ib and Ib′ had decreased absorption at 3360 and 1020 cm^{-1}, compared to the spectra of Ia and Ia′, showing the loss of a hydroxyl group; however, an absorption at 3430 cm^{-1} (sharp) and 3340 cm^{-1} (broad) indicated a free amido group. Absorption at 975 cm^{-1} was evidence of a *trans* double bond (see Table II).

NMR decoupling experiments established relative positions of the functional groups. Decoupling results were identical for both Ia and Ia′. Irradiation of either proton-7 or -5 (Table III) at δ 4.12 or δ 3.74, respectively, decoupled olefinic absorption. Decoupling of proton-4 had no visible effect on spectra; proton-5 could not be observed because it was obscured by protons-13. Irradiation of proton-10 collapsed the doublet of doublets for each of the geminal protons-9 to a doublet and, thus, simplified analysis of the cluster of absorptions at about δ 3.3.

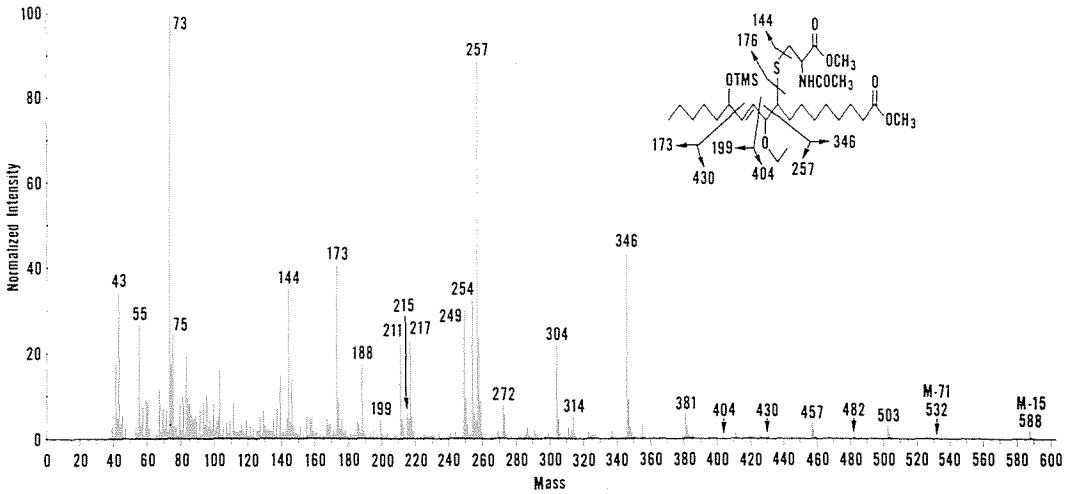
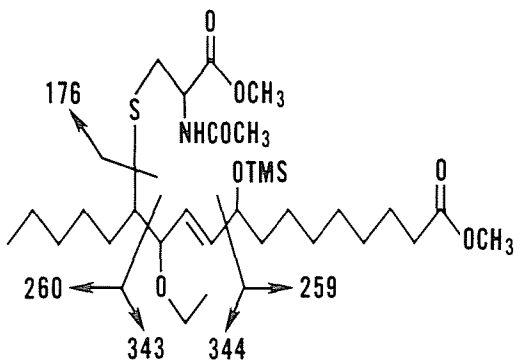


FIG. 2. Mass spectrum of Ib. See Table IV for subtraction ions.

Spectral differences between Ia and Ia′ were insignificant, except for the NMR analyses. The most pronounced differences in Ia and Ia′ NMR absorptions were protons-6, -9, and -10 (Table III). There were other subtle differences inasmuch as NMR analyses of mixtures of Ia and Ia′ revealed that the absorption of protons-15 was a split triplet, absorption of protons-13 was a split singlet, and absorption of protons-14 was more complex than a simple quartet. Possibly the minor differences in chemical shift, reported in Table III, for these protons are not experimental errors. There is no obvious reason for the difference in Ia and Ia′ other than *erythro-threo* isomerism about carbons 9 and 10 of the fatty chain. Conceivably, perturbation of the molecular environment near these groups causes the minor differences in NMR spectra.

MS spectrum of Ib (same as Ib′) in Figure 2 established that the TMS group was at carbon 13 of the fatty ester chain and that the *N*-acetylcysteinethio methyl ester group was at carbon 9. The position of the other groups was confirmed as well.

As additional structural evidence, a mixture of LOOH isomers, 21% 9-LOOH and 79% 13-LOOH, was used as a reactant with *N*-acetylcysteine. As expected, I and I′ from this reaction were mixtures of positional isomers (79% 9-*S*-[*N*-acetylcysteine]-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid and 21% 13-*S*-[*N*-acetylcysteine]-9-hydroxy-12-ethoxy-*trans*-10-octadecenoic acid). An MS spectrum of Ib′, which had originated from this reaction, had fragment ions characteristic of both isomeric forms. In addition to the ions shown in Figure 2, the ions *m/e* 259, 260, 343, and 168 (344-176) were observed between 10 and 20% relative intensity, as shown in the following structure:



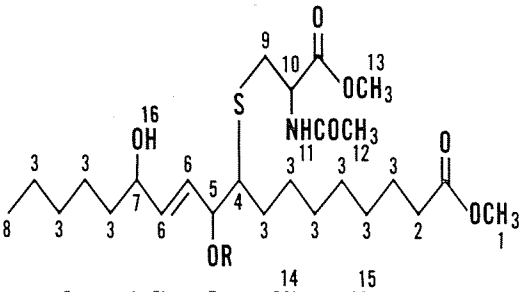
Products II and II′: Products II and II′ were isolated as their methyl esters, IIa and IIa′. Column chromatography separated IIa and IIa′ from the mixture (Table I).

Spectral results for IIa and IIa′ are shown in Tables II and III. The MS spectrum of IIb (Fig. 3) is identical to the MS of IIb′ within experimental error. Table IV explains the subtraction ions from Figure 3. These data and additional experiments outlined below were used to investigate the structure.

Two hydroxyls were indicated by NMR analysis of the derivative, IIb′. Absorptions at δ 0.11 (s, 9 H) and δ 0.13 (s, 9 H) were due to two TMS groups. Compared to the IR of IIa and IIa′, IR spectra of IIb and IIb′ had greatly decreased absorptions at 3350 and 1020 cm^{-1}, indicating the loss of hydroxyl groups; absorp-

TABLE III

Nuclear Magnetic Resonance Spectra of Addition Products and Their Respective Isomers[a]



Proton	δ (Multiplicity, J, Hz)			
	Ia	Ia′	IIa	IIa′
1 (3H)	3.64(s)[b]	3.64(s)	3.66(s)	3.66(s)
2 (2H)	2.29(t)[b]	2.29(t)	2.30(t)	2.30(t)
3 (20H)	1.28(m)[b]	1.28(m)	1.30(m)	1.30(m)
4 (1H)	2.72(m)	2.73(m)	2.74(m)	2.76(m)
5 (1H)	Obscured by 13	Obscured by 13	4.16(m)	4.19(m)
6[c] (2H)	5.66(m)	5.69(m)	5.74(m)	5.74(m)
7 (1H)	4.11(m)	4.13(m)	4.16(m)	4.19(m)
8 (3H)	0.87(t)	0.87(t)	0.88(t)	0.88(t)
9 (2H)	2.95(dd,5,14)[b]	2.88(dd,7,13)	2.90(dd,5,14)	2.91(dd,6,14)
	3.18(dd,5,14)	3.24(dd,6,13)	3.11(dd,5,14)	3.20(dd,6,14)
10 (1H)	4.78(m)	4.71(m)	4.88(m)	4.80(m)
11 (1H)	6.81(d)[b]	6.51-6.60(d)	6.82-6.87(d)	6.45-6.85(d)
12 (3H)	2.02(s)	2.02(s)	2.04(s)	2.05(s)
13 (3H)	3.74(s)	3.73(s)	3.76(s)	3.79(s)
14 (2H)	3.42(q)[b]	3.43(q)[d]	---	---
15 (3H)	1.18(t)	1.17(t)	---	---
16[e]				

[a]Ia′ and IIa′ are isomeric forms of Ia and IIa postulated to be *erythro* and *threo* about carbons 9 and 10 of the fatty ester chain.

[b]d = doublet, dd = doublet of doublet, m = multiplet, q = quartet, s = singlet, t = triplet.

[c]The absorption differed between Ia and Ia′ in the spacing of the peaks: Ia δ 5.69, 5.66, and 5.62; Ia′ δ 5.73, 5.68, and 5.66. Because the absorptions of protons-6 were compact, J could not be measured.

[d]Some hyperfine splitting observed.

[e]The chemical shift was variable and broad. Usually, absorption was difficult to locate and was more visible in spectra of IIa and IIa′ than of Ia and Ia′.

tions at 3430 cm^{-1} (sharp) and 3340 cm^{-1} (broad) were due to a free amido group. IR absorption at 975 cm^{-1} indicated the presence of a *trans* olefin (see Table II).

Relative positions of the functional groups were ascertained by NMR decoupling experiments. Results were identical for IIa and IIa′. Irradiation of protons-5 and -7 (Table III) centered at δ 4.16 (4.19) decoupled the olefinic absorption. Irradiation of proton-4 at δ 2.74 (2.76) affected the downfield portion of the absorption at δ 4.16 (4.19), which was undoubtedly due to decoupling of proton-4 from proton-5. Because the upfield portion of the absorption centered at δ 4.16 (4.19) was unaffected by the decoupling experiment, this absorption is probably due to proton-7.

Slight differences in the NMR spectra of IIa and IIa′ were indicative of isomerism as observed for Ia and Ia′ (Table III). The most striking difference was the absorption of the nonequivalent geminal protons-9. Whereas the absorption of these protons was well separated as two doublets of doublets in the spectrum of IIa′, a smaller difference in chemical shift made the two innermost peaks in the IIa spectrum

TABLE IV

Subtraction and Rearrangement Ions Observed in Mass Spectra of Ib and IIb

Observed ion (*m/e*)	Subtracted from parent ion[a]	
	Ib	IIb
211	257-46	301-90
215	404-(176 + 45)	
216	404-(176 + 46)	
217	249-32	249-32
249	176 + 73 R	176 + 73 R
254	430-176	
272	346-(43 + 31)	346-(43 + 31)
298		474-176
304	346-43 + 1	346-43 + 1
314	346-32	346-32
419		346 + 73 R
422		576-(90 + 32 + 32)
482	M - (90 + 31)	
503	M - 173 + 73 R	
526		M - (90 + 31)

[a]Moieties subtracted are 176, 173, and 144, see Figure 3; 43, acetyl; 31 or 32, ester methoxy; 45 or 46 ether ethoxy; 90, trimethylsilyloxy + 1; and 73, trimethylsilyl. R represents a rearrangement ion according to Kleiman and Spencer (17).

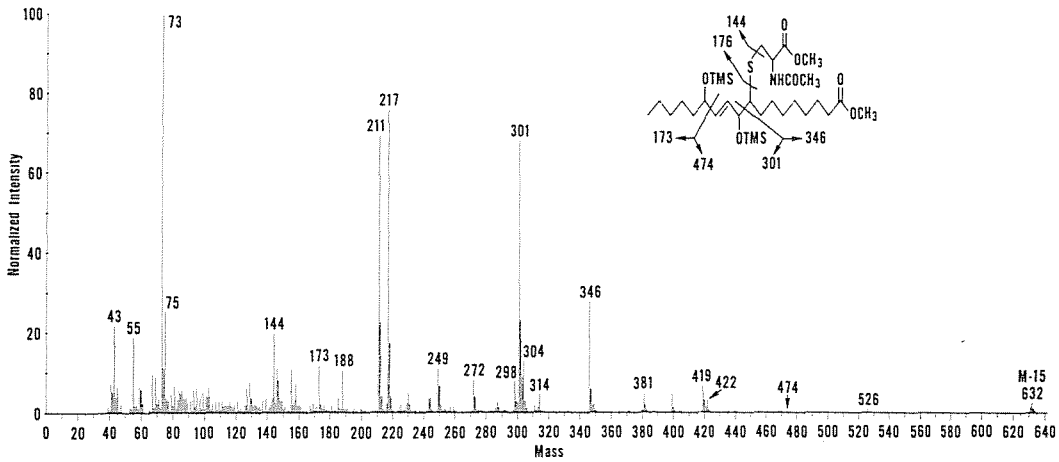
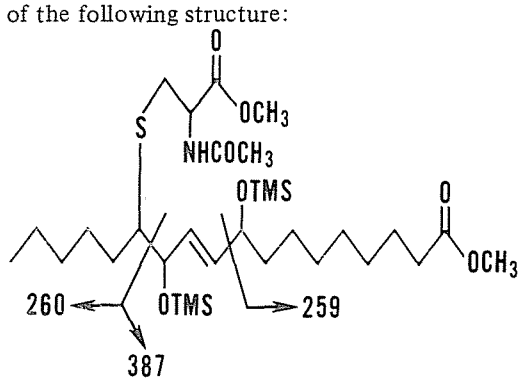


FIG. 3. Mass spectrum of IIb. See Table IV for subtraction ions.

overlap. Decoupling proton-10 resolved the overlap. Because most of the observed differences in IIa and IIa′ are centered about the assymetric center of carbons 9 and 10 of the fatty ester chain, *erythro-threo* isomerism is indicated but not proved.

The MS spectrum of IIb (same as IIb′) appears in Figure 3. TMS groups are indicated for carbons 13 and 10 on the fatty ester chain, and an *N*-acetylcysteinethio methyl ester group at carbon 9.

As was shown for Ia and Ia′, additional evidence was obtained by using mixed hydroperoxides, 21% 9-LOOH and 79% 13-LOOH, as reactants with *N*-acetylcysteine. IIb′ derived from this reaction was a mixture of positional isomers. Besides obtaining the fragment ions in Figure 3, ions at 259, 260, 387, 333 (260 + 73R), and 297 (387-90) *m/e* with relative intensity between 5-10% indicated the presence of the following structure:



N,N'-Diacetylcystine: *N,N'*-Diacetylcystine dimethyl ester was isolated from the product mixture by column chromatography followed by TLC. Final purification was by crystallization from ether-methanol. The isolated material yielded an IR spectrum ($CHCl_3$) with characteristic absorptions due to a monosubstituted amide at 3430, 3320, 1665, and 1500 cm^{-1}, and those due to an ester carbonyl at 1740 cm^{-1}. NMR gave the following absorptions: acetyl, δ 2.04 (s, 6 H); methylene, δ 3.16 (d, 4 H); ester methoxy, δ 3.73 (s, 6 H); amide methine, δ 4.81 (m, 2 H); and amide, δ 7.09 (d, 2 H). An MS spectrum had the following intense ions: M(*m/e* 352), M - 59, 176 (disulfide cleavage), 144 (176-32), 134, 118, 102, 88, 84, 60, and 43.

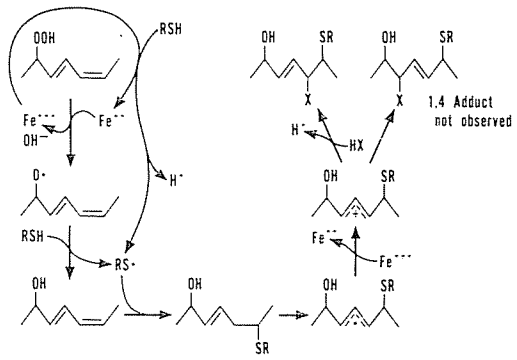


FIG. 4. Pathway proposed for formation of addition products. HX, solvent; RSH, *N*-acetylcysteine; fatty acid structures are abbreviated.

DISCUSSION

As a reactant, *N*-acetylcysteine was selected as a derivative of cysteine that would more closely resemble a model of this amino acid in a protein. Some of our unpublished results (18) indicate that *N*-acetylcysteine and cysteine behave similarly regarding the addition reaction described here, but that cysteine also yields products from a browning reaction which is probably due to Schiff base formation between the amino group and the aldehydes produced as secondary products of LOOH decomposition (19). Additionally, since metal ions form bidentate complexes with the thiol and amino groups of cysteine (20), using *N*-acetylcysteine demonstrated that the presence of an amino group is not necessary for the reaction to take place. The presence of 10^{-5}M ionic iron is necessary to complete the reaction within the time allotted. Without catalyst, no significant reaction was observed. Generally, complexes of cysteine and Fe(III) undergo an intramolecular electron shift from sulfur to a vacant *d*-orbital of iron, giving an Fe(II) species complexed with a thiyl radical (20). Taylor et al. (21) indicated there was also a possibility for an ionic mechanism.

Thiyl radicals are indicated because *N*-acetylcysteine added to the conjugated diene by anti-Markownikoff rule (12). Thiyl radicals additionally were indicated by production of the disulfide, *N,N'*-diacetylcystine. After thiyl addition to the conjugated diene, an available hydrogen radical usually adds at either end of the resultant allyl radical. Instead, an oxygenated function added; i.e., hydroxy or ethoxy. In a previous study (13) of this reaction, it appeared that hydrogen radicals were unimportant in addition reactions, but rather doubly and triply oxygenated fatty acids resulted. In formation of products II and II', hydroxyl could add as a result of the expected presence of hydroxy radicals with Fenton reagents (22). Conditions for Fenton reactions presumably are possible through interaction of Fe(II) and hydroperoxide. However, addition of an ethoxy group (products I and I') presents a mechanistic problem. The energetics of ethoxy radical formation are usually unfavorable. Although transition metal ions often catalyze alkoxy radical formation from highly substituted alcohols, primary alkanols are usually converted into carbon-centered radicals (23). Instead, solvolysis is indicated in which water (II and II') and ethanol (I and I') have participated in a substitution reaction. Substitution can occur by metal ion oxidation of alkyl radicals to carbonium ions (24), and this appears to be responsible for the origin of the hydroxyl or ethoxy group α to the *N*-acetylcysteinethio group. A possible pathway is shown in Figure 4. In metal-catalyzed oxidation of allylic radicals, 1,2 adducts always predominate over 1,4-adducts because the metal ion coordinates with the Π-bond of the allylic radical (25); consequently, the absence of the 1,4 addition products in this study is explained.

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